ISOLATION OF YEAST STRAINS WITH ABILITY TO REDUCE VOLATILE ACIDITY OF WINES

Vilela-Moura A. [(1)], Schuller D. [(2)], Mendes-Faia A. [(1)] and Côrte-Real M. [(2*)]
(1) CGB/IBB, Universidade de Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal
(2) Centro/Departamento de Biologia, Universidade do Minho, 4710-057 Braga, Portugal
*For correspondence: Manuela Côrte-Real, e-mail: mcortereal@bio.uminho.pt

Abstract

From a set of 135 yeasts isolated during a wine cellar refermentation procedure, four strains revealed ability to use glucose and acetic acid simultaneously in solid media. Three of them were identified as *Saccharomyces cerevisiae* and one as *Lachancea thermotolerans* by sequencing the D1/D2 domain of the large subunit (26S) ribosomal DNA. Under limited aerobic conditions and in two culture media containing acidic wines with high glucose/low ethanol or low glucose/high ethanol concentrations, the *S. cerevisiae* isolates show simultaneous consumption pattern of glucose and acetic acid and acid removal efficiencies identical to *Zygosaccharomyces bailii* ISA1307. A more widespread analysis of different *S. cerevisiae* strains would be useful to determine how general this phenomenom is.

Introduction

Acetic acid is the main component of volatile acidity, and is critical for wine quality. Its concentration in wines is approximately 0.5 gl^{-1}, and legally, must remain below 0.1% (w/v). This acid is mainly produced by bacterial spoilage in *Botrytis cinerea* infected grapes. Acetic acid can also be formed by yeasts during alcoholic fermentation. *S. cerevisiae* is a yeast species that can use acetic acid as a sole carbon and energy source. During growth in acetic acid containing media, this substrate is metabolized via acetyl coenzyme A which supplies lipid biosynthesis and other metabolic precursors through Krebs cycle and gluconeogenesis [1]. Winemakers have been using an empirical biological deacidification procedure in order to lower acetic acid contents of wines with high volatile acidity (higher than 0.8 gl^{-1}) and which consists in a refermentation associated to acetic acid consumption by yeasts. According to Ribéreau-Gayon and co-workers [2] this enological practice is performed by mixing the acidic wine with freshly crushed grapes or musts in a proportion of no more than 20-30% (v/v). The initial volatile acidity of this mixture should not exceed 0.6 gl^{-1}, and the final volatile acidity of the newly made wine rarely exceeds 0.3 gl^{-1}. Alternatively, the acidic wine can be incubated with the residual marc from a finished wine fermentation. The aim of the present study was to isolate and characterize indigenous yeasts species from typical refermentation processes that can be used as starters in an efficient and controlled biological procedure to decrease volatile acidity of acidic wines.

Materials and Methods

Microorganisms

During a refermentation process of a wine with excessive acetic acid concentration (1.36 gl^{-1}) 135 yeast isolates were collected. From this group, four wild strains (43C, 45C, 44C and 30C) were selected based on their ability to consume acetic acid in the presence of glucose in a solid media, at pH 4.0 or 6.0, as described results. The strains *Z. bailii* ISA 1307 and *S. cerevisiae* IGC 4072 were used as control strains.

Culture media and growth conditions

Strains were kept at -80ºC in micro tubes containing YPD broth (glucose 2% w/v, peptone 1% w/v, yeast extract 0.5% w/v) supplemented with glycerol (30%, v/v). Acetic acid utilization was assessed in a minimal mineral medium [3] containing acetic acid (0.5% v/v) and glucose (from 0.5% w/v up to 5% w/v) at 25ºC and pH 3.0. Acetic acid and glucose consumption was evaluated under aerobic and limited-aerobic conditions (250 ml Erlenmeyer flask containing 100 ml of

minimal medium (120 rpm) and containing 230 ml of minimal medium (100 rpm), respectively). In order to simulate a refermentation of a wine with excessive amounts of acetic acid, mixtures were prepared containing 2/3 of a mineral medium [3] and 1/3 of acidic white wine. Volatile acidity of the mixture was adjusted to 1.12 - 1.14 gl^{-1}, using glacial acetic acid (Merck), and the pH of the medium to 3.5, using NaOH. The medium containing wine 1 (ethanol, 4% v/v) was further supplemented with glucose (13 %, w/v) in order to simulate a refermentation of an acidic wine with must from the beginning of fermentation. The mineral medium containing wine 2 (ethanol, 10% v/v) was supplemented with glucose (3.3% w/v) and in order to simulate a refermentation of an acidic wine with the residual marc from a finished wine fermentation. Ten ml of the inoculated media were incubated overnight and used as a pre-culture for growth experiments, at 25ºC, under both aerobic and limited-aerobic conditions, as described before. Growth was monitored by measuring absorbance at 640 nm (UV-VIS Scanning Spectrophotometer, Shimadzu UV-2101PC).

Analytical determinations

Glucose, acetic acid, ethanol and lactic acid concentrations were determined by HPLC after cells separation by filtration. A Perkin-Elmer series 10 Liquid Chromatographic System equipped with an ion exclusion, cation exchange column (BIORAD – HPX – 87H) and an RI detector was used. The column was eluted with sulfphuric acid (0.013N) at 62ºC, at flow rate of 0.6 ml/min. The components were identified by their relative retention times, which were determined by injection of standards. Acetic acid concentration was also determined enzymatically with an Enzytec Laboratories kit. The values presented are averages of triplicates.

Molecular identification (D1/D2 region amplification and sequencing)

Identification was based on ribosomal DNA gene sequence analysis. The D1/D2 variable domain at the 5' end of the 26S rDNA (nucleotides 63–642 for *S. cerevisiae*) was amplified with primers NL-1 (GCATATCAATAAGCGGAGGAAAAG) and NL-4 (GGTCCGTGTTTCAAGACGG) [4], obtained from MWG Biotech, Germany. PCR were performed and the amplified fragments were purified (Nucleospin Extract II PCR cleanup kit, Machery-Nagel) and sequenced by using the ABI Big Dye Terminator kit (version 3.1). Processing of the samples for loading on to ABI 310 model sequencer was performed as per manufacturer's instructions. Sequence similarity search was done using GenBank BLASTN search [5].

Results and discussion

Study of isolated wine strains regarding their ability to degrade acetic acid

Aiming to select yeast species with ability to remove volatile acidity from grape musts or wines, 135 yeast isolates were collected during a refermentation process of acidic wines, carried out in a wine cellar. The strains were tested regarding their growth patterns in a differential medium [6] containing glucose (0.2%, w/v) and acetic acid (0.5%, v/v), at pH 4.0 or 6.0 (data not shown).The selected strains 43C, 44C, 45C and 30C displayed growth associated to color change of the pH indicator of the medium indicative of simultaneous glucose and acetic acid consumption. Subsequently, the effects of glucose and acetic acid concentrations, as well as aeration conditions on the consumption of acetic acid by the four isolates were studied. The strains *Z. bailii* ISA1307 and *S. cerevisiae* IGC 4072, previously described to display respectively, a simultaneous [7] and sequential consumption of glucose and acetic acid [8], were used as references. A one-way ANOVA, or single factor ANOVA (Excel, Microsoft) was used to evaluate the differences between the yeasts strains concerning acetic acid consumption in the different assays.

Though associated with significantly different acetic acid consumption rates ($P \leq 0.05$) all the strains tested, excepting *S. cerevisiae* IGC 4072, were able to exhaust acetic acid from the medium, under aerobic conditions and for 0.5% (w/v) of glucose (Table 1). In fact, *Z. bailii* ISA 1307 was the faster strain to remove acetic acid (after 72h) followed by the isolates 43C and 45C (168h), 30 C (192h) and 44C (216h). Oxygen limitation and a slight increase in glucose concentration (0.75%,

w/v) led to a decrease of the acid consumption rates by all strains excepting *Z. bailii* ISA 1307. Under these conditions *Z. bailii* ISA 1307 and isolate 43C behaved significantly different ($P \leq 0.05$) and degraded about 100 and 60 % of the initial acetic acid after 312h, respectively, whereas the other strains displayed low acid removal percentages (Table 1). Further increase in glucose concentration up to 5% (w/v), under limited aerobic conditions, reduced the acid removal to about 60% after 312h. Isolates 30C, 43C and 45C did not displayed visible acid consumption. Curiously, isolate 44C albeit not being able to exhaust glucose after 312h, displayed ability to remove about 28% of the acid.

Table 1 - Consumption of acetic acid and glucose by the four yeast isolates in comparison with *S. cerevisiae* strains S26, and *Z. bailii* ISA 1307, in minimal media (pH 3.0) with different initial concentrations of glucose (0.5% to 5% w/v) and acetic acid (0.5% v/v), under aerobic and limited-aerobic conditions.

Yeasts strains	Aerobic conditions		Limited-aerobic conditions			
	Glucose (0.5% w/v)		Glucose (0.75% w/v)		Glucose (5% w/v)	
	Glucose (gl^{-1})	Acetic acid (gl^{-1})	Glucose (gl^{-1})	Acetic acid (gl^{-1})	Glucose (gl^{-1})	Acetic acid (gl^{-1})
ISA 1307	0	0 (72 h)*	0	0.02+….	0	1.92
IGC 4072	0	4.0 (216)	0	3.00+	0	4.96
30C	0	0 (192 h)*	0	4.40	0	4.90
43C	0	0 (168 h)*	0	2.02	0	4.77
44C	0	0 (216 h)*	0	3.99	15.11	3.59
45C	0	0 (168 h)*	0	4.01	0	4.71

*Time needed to exhaust acetic acid from the medium. Pode-se acrecentar o desvio padrão???

Molecular identification of the isolated wine strains

D1/D2 sequence of strain 30C, 43C and 45C showed 99-100% identity with deposited *S. cerevisiae* sequences (accession numbers U53879, AY130346 and U44806, respectively). D1/D2 sequence of strain 44C shows 99% of identity with that of strain *Lachancea thermotolerans* NRRL Y-8284 (accession number U69581) [9,10].

Refermentation simulation assays of acidic wines with the selected wine yeast strains

The two *S. cerevisiae* isolates (43C and 45C), the *L. thermotolerans* isolate (44C) and the strain *Z. bailii* ISA 1307 were further tested under conditions simulating refermentation processes. Under aerobic conditions and in the presence of high glucose and low ethanol initial concentration, all strains consumed acetic acid simultaneously with glucose. However, *Z. bailii* ISA 1307 followed by *S. cerevisiae* 43C and 45C were the faster strains removing respectively 91%, 17.5% and 16.7% of the acid, after 48h (Table 2). *L. thermotolerans* 44C, behaved similarly to *Z. bailii* ISA 1307, being able to exhaust the acid from the medium though after a much longer incubation period. Under limited aerobic conditions there were no differences in percentage of acid removal between *Z. bailii* ISA 1307 and the *S. cerevisiae* isolates 43C and 45C, after 48h. This was due to a descrease in acid removal for *Z. bailii* ISA 1307 and to an increase for *S. cerevisiae* 43C and 45C indicating that oxygen limitations affected inversely the efficiency of each species (Table 2). After 48h *L. thermotolerans* 44C displayed no visible acetic acid and glucose consumption associated to an extended lag phase under oxygen limitations conditions (not shown). The slower growth of *L. thermotolerans* 44C is consistent with its less tolerance to low oxygen availability than *S. cerevisiae* strains [11].

Regarding refermentation assays with acidic wine containing media with low glucose and high ethanol initial concentrations (Table 2), the strain *Z. bailii* ISA 1307 appears again faster than *S.*

cerevisiae isolates 43C and 45C with an acid removal after 72h of about 50%, comparatively to about 30%, respectively.

Table 2 – Comparison of acetic acid (A) and glucose (G) consumption (%) for each strain tested in the refermentation simulation assays, after a given incubation time (T), and maximum values of acetic acid consumption achieved (A_{max}) and correspondent glucose consumption (GA_{max}) at given incubation times (T_{max}).

	Glucose 13% (w/v) and ethanol 4% (v/v)								Glucose 3.3% (w/v) and ethanol 10% (v/v)							
	Aerobic conditions				Limited-aerobic conditions				Aerobic conditions				Limited-aerobic conditions			
Yeast strains	A G	T (h)	A_{max} GA_{max}	T_{max} (h)	A G	T (h)	A_{max} GA_{max}	T_{max} (h)	A G	T (h)	A_{max} GA_{max}	T_{max} (h)	A G	T (h)	A_{max} GA_{max}	T_{max} (h)
ISA 1307	91.2 35.6	48	91.2 35.6	48	52.6 8.2	48	67.5 88.8	168	52.7 4.3	72	96.4 61.8	120	29.5 11.7	72	91.9 100	408
43C	17.5 86.1	48	34.2 98.2	72	53.5 71.1	48	53.5 71.1	48	33.0 100	72	33.0 100	72	34.8 83.9*	72	34.8 83.9	72
44C	6.1 1.8	48	99.1 98.0	264	0 0	48	35.1 2.3	168	18.8 4.9	72	83.9 100	336	22.3 8.3	72	48.2 100	408
45C	16.7 90.1	48	16.7 90.1	48	52.1 61.4	48	52.1 61.4	48	28.6 100	72	28.6 100	72	34.8 74.6*	72	34.8 74.6*	72

* These strains completely depleted glucose from the medium after 96 h.

This observation indicates that while glucose/ethanol concentration affects acid removal by *Z. bailii* ISA 1307 it does not affect acid removal by *S. cerevisiae* 43C and 45C (Table 2). As observed for high glucose and low ethanol concentrations oxygen limitation reduced the percentage of acid removal by *Z. bailii* ISA 1307.Yet, the percentages of acid removal by *S. cerevisiae* 43C and 45C under these low glucose and high ethanol concentrations appear not affected by oxygen limitations. Therefore, under these latter conditions *S. cerevisiae* 43C and 45C appear equally efficient as *Z. bailii* ISA 1307. *L. thermotolerans* 44C only reached considerable values of removal of acetic acid after much longer periods, both under limited and non-limited aerobic conditions. Considering that *Z. bailii* is undesirable for enological applications and that, from the perspective of practical implementation the limited aerobic conditions are more realistic, the data obtained show that *S. cerevisiae* isolates can be used to decrease the volatile acidity of acidic wines. A more widespread analysis of different *S. cerevisiae* strains will be done to determine how general the phenomenon is.

Acknowledgements

Part of this study was supported by the programme POCI 2010 (project POCI/AGR/56102/2004).

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